Mol. Cells *31*, 151-158, February 28, 2011
DOI/10.1007/s10059-011-0012-z

Molecules and Cells

Effect of *Saccharomyces cerevisiae ret1-1* Mutation on Glycosylation and Localization of the Secretome

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To study the effect of the *ret1-1* mutation on the secretome, the glycosylation patterns and locations of the secretory proteins and glycosyltransferases responsible for glycosylation were investigated. Analyses of secretory proteins and cell wall-associated glycoproteins showed severe impairment of glycosylation in this mutant. Results from 2D- polyacrylamide gel electrophoresis (PAGE) indicated defects in the glycosylation and cellular localization of SDS-soluble cell wall proteins. Localization of RFP-tagged glycosyltransferase proteins in *ret1-1* indicated an impairment of Golgi-to retrograde transport at a non-permissive temperature. Thus, impaired glycosylation caused by the mislocalization of ER resident proteins appears to be responsible for the alterations in the secretome and the increased sensitivity to ER stress in *ret1-1* mutant cells.

INTRODUCTION

The cell wall is essential for the survival and osmotic integrity of fungal cells such as *Saccharomyces cerevisiae*. Electron microscopic analysis of the cell wall reveals a structure with inner and outer layers. The mechanical strength of the wall is mainly provided by the inner layer, which consists of β-1,3-glucan and chitin. The outer layer, which consists of heavily glycosylated mannoproteins with a large amount of *N*- and/or *O*-linked mannoses, is involved in biological functions such as cell-cell recognition and protection from cell wall-degrading enzymes (Cappellaro et al., 1994; Osumi, 1998). Glycosylation is an important modification that regulates the structure and function of secreted and membrane proteins. Proteins are passed into the endoplasmic reticulum (ER) cotranslationally, glycosylated, and are sent to the Golgi apparatus for further processing; they are subsequently targeted either to various organelles, along the secretory pathway, become plasma membrane components, or are secreted into the periplasm (Kapteyn et al., 1997).

In *S. cerevisiae*, the transfer of a preassembled high mannose-containing oligosaccharide onto nascent polypeptides is catalyzed by the 9 subunit oligosaccharyltransferase (OST) complex (Ost1p-Ost6p, Stt3p, Swp1p, and Wbp1p) in the lumen of the rough ER (Herscovics and Orlean, 1993). Defects in the transfer and modification of *N*-linked oligosaccharides cause biochemical and structural alterations in yeast cell walls (Klis, 1994). Cell wall defects in strains deficient in genes responsible for the *O*-mannosylation of proteins (*PMT1-PMT7*) indicate that this type of modification is also required for cell wall rigidity and cell integrity (Strahl-Bolsinger et al., 1999). In yeasts, protein *O*-glycosylation is initiated in the ER by protein mannosyltransferases (PMTs). Pmt2p interacts with Pmt1p, which is a key enzyme of protein *O*-glycosylation; the phenotypes of strains bearing a *pmt2* deletion are similar to those of the *pmt1* strain (Gentzsch and Tanner, 1997). Since cell wall mannoproteins are modified with a large amount of *N*- or *O*-linked mannose (Klis, 1994), the amount of mannose in cell wall proteins reflects the degree of glycosylation.

The COPI coat consists of the GTP-bound ADP ribosylation factor (ARF) and coatomer, which comprises 7 subunits: α-, β-, β′-, γ-, δ-, ε-, and ζ-COP; except for ε-COP, each of these COP proteins is essential for yeast cell viability. Biochemical analysis using an *in vitro* protein transport assay suggests that COPI vesicles mediate *cis*- to *medial*-Golgi protein transport (Ostermann et al., 1993; Waters et al., 1991) and the recycling of ER resident proteins from the pre-Golgi compartment back to the ER (Cosson and Letourneur, 1994). Among the mutant alleles of *RET1* (α-COP), the *ret1-1* mutation impairs the retrograde transport of dilysine-tagged ER membrane proteins such as Wbp1, which is an essential subunit of the OST complex (Letourneur et al., 1994; Schroder-Kohne et al., 1998).

We previously reported that the *soo1-1* and *ret1-1* mutations are identical. The *ret1-1* mutation is responsible for the thermosensitive osmo-remediable phenotype and impairs the post-translational modification and proper localization of cell wall proteins (Kim and Park, 2004; Lee et al., 1999; 2002). These results suggest that Ret1p/α-COP play a crucial role in the recognition and translocation of ER resident proteins responsible for post-translational modification; thus, Ret1p/α-COP may contribute to the maintenance of cell wall integrity and biogenesis in *S. cerevisiae*. However, little is known about Ret1p or its role in the post-translational modification and secretion of proteins destined for the cell surface. In this study, we investigated the effect of the *ret1-1* mutation on the location of the ER-resident glycosyltransferases responsible for the glycosylation

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Received August 30, 2010; revised October 30, 2010; accepted November 1, 2010; published online November 25, 2010

Keywords: glycosylation, localization, *ret1-1* mutation, *Saccharomyces cerevisiae,* secretome

of secretory proteins and consequently the glycosylation patterns of the exoproteome. We also tested the ER-stress response phenotype of the *ret1-1* mutant using unfolded protein response (UPR)-inducing agents.

MATERIALS AND METHODS

Strains and culture conditions

S. cerevisiae strains LP0353 and LP0353RS1 were used in this study under previously described culturing conditions (Kim and Park, 2004). The yeast strains were grown in yeast extract/peptone/dextrose (PD) medium (1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose) or synthetic minimal medium, (SD; 0.67% Bacto-yeast nitrogen base without amino acids and 2% dextrose). D-sorbitol was added at a concentration of 1.2 M as an osmotic stabilizer when the cells were grown at the non-permissive temperature of 37°C. *E. coli* strains DH5α and BL21 (DE3) were used for the propagation and preparation of recombinant plasmids, and the expression of antigen proteins, respectively.

Red fluorescent protein (RFP) tagging

Strains with integrated RFP tags on endogenous genes for ER resident proteins were constructed using previously described methods (Huh et al., 2003). Strains with RFP-tagged proteins were analyzed by fluorescence microscopy (LSM 510; Carl Zeiss Co., Germany). Primers for the construction of RFP-fusion genes are listed in Table 1.

Fusion protein and antibody preparation

Anti-Cwp1p antisera were supplied by Dr. Shimoi et al. (1998), and HA IgG was purchased from GE healthcare (USA). To construct the expression cassettes for Cts1p and Gas1p, parts of each open reading frame were amplified by PCR using yeast total genomic DNA and primers linked with *Bam*HI and *Eco*RI respectively (Table 1). The PCR products were cloned into the *Bam*HI- and *Eco*RI-digested pGEX-4T vectors (GE healthcare, USA) to yield pGEX-4T-Cts1p and pGEX-4T-Gas1p respectively. The expression of the glutathionine *S*-transferase (GST)-fused proteins was induced as described previously (Smith and Johnson, 1988). Antisera against fusion proteins and carboxypeptidase Y (Sigma, USA) were prepared as described previously (Kim and Park, 2004).

Preparation of cell wall proteins

Cell wall proteins were prepared as described previously (Kim and Park, 2004; Shimoi et al., 1995; 1998). Cells were inoculated into media and precultured at 28°C overnight. After the addition of 1 ml preculture to 100 ml main culture in a 500 ml culture flask, the cells were incubated at permissive or non-permissive temperatures with shaking at 220 rpm in a rotary shaker until the OD_{600} reached 1.0. The cells were harvested by centrifugation, washed, and disrupted with glass beads in a bead beater (Biospec Products., USA). The glass beads were removed by decantation; the cell walls were pelleted by centrifugation at 1,500 $\times$ *g* for 5 min and washed with 5 M LiCl 5 times. For the preparation of cell wall proteins, the cell walls were suspended in 0.5 ml sodium dodecyl sulfate (SDS) extraction buffer (50 mM Tris-HCl (pH 8.0), 2% SDS, 100 mM EDTA, and 40 mM dithiothreitol); the cell walls were then heated at 100°C for 10 min to harvest the SDS-extractable cell wall proteins that were non-covalently bound or bound by disulfide bridges. The SDS-treated cell walls were pelleted by centrifugation at 1,500 $\times$ *g* for 5 min, washed with 1 mM phenylmethylsulfonyl fluoride 5 times, once with 50 mM Tris-HCl buffer (pH 8.0), and digested with 20 units/ml Quantazyme (Qbiogene, Montreal, Canada) in 0.2 ml reaction buffer (67 mM KH_2PO_4 (pH 7.5) and 120 mM β-mercaptoethanol) at 30°C for 6 h. The reaction mixture containing solubilized proteins was centrifuged at 15,000 $\times$ *g* for 10 min, and the supernatant was analyzed as β-1,3-glucanase-extracted cell wall proteins.

Table 1. Oligonucleotides used in this study

Fragments	Sequence of oligonucleotide (5′ to 3′)
Cts1p	CGGGATCCGAAGTAGGCTATAGTGCG/ GAATTCCGGAAAAGGCTTGAGATGC
Gas1p	CGGGATCCATGTTGTTTAAATCCCTTTCA/ GGAATTCCTGCAGTTCTATCAGCATAA
Wbp1-RFP tag	ACGACTTCCTCTGTTGGCAAGAAACTTGAAACATT CAAAAAAACAAACATGGCCTCCTCCGAGGACGT/ ATGTGAGCCATTTATAAAGAATTATAAAGGATATG AAAACATATATTATGGGCAGATGATGTCGAGGCG AAAAAAAAT
Pmt2-RFP tag	TACTTAAACTGGTTTTCCACTTGGGACATTGCCGA CAAGCAAGAAGCAATGGCCTCCTCCGAGGACGT/ TTAATTCTTGTTTTTTACTTATGTACTTACTACGAT GATTTTAATTCATGGGCAGATGATGTCGAGGCGA AAAAAAAT

Western blotting

Protein samples were subjected to SDS-PAGE using the method described by Laemmli (1970) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane in a solution of 25 mM Tris, 192 mM glycine, 20% methanol, and 0.05% SDS using Semiphore transfer units (Amersham). The PVDF membrane was first saturated with blocking buffer (0.02% Tween 20 and 5% skim milk in PBS) for 1 h. Primary antibodies (rabbit anti-Cwp1p, and mouse anti-HA, anti-Cts1p, and anti-Gas1p) were used to probe the membranes. Reactions were developed with anti-rabbit and anti-mouse immunoglobulin G antibodies conjugated with horseradish peroxidase for anti-Cwp1p and other mouse antibodies respectively (Amersham). Immunoblotting was carried out using the enhanced chemiluminescence (ECL) method using the ECL labeling and detection kit (Amersham) as described in the manufacturer's manual (Kim and Park, 2004).

Invertase activity staining

For invertase activity staining, cell lysates were prepared using YeastBuster (Novagen Co., Germany) and separated using a 6% Gradi-Gel™ I (Elpis-biotech Co., Korea). The gel was soaked in 50 ml cold sucrose solution buffer (0.1 M sucrose and 0.1 M CH_3COONa; pH 5.1) and then incubated for 30 min at 4°C with shaking. The gel incubated in cold buffer was transferred to sucrose solution buffer at 37°C and subsequently incubated for 1 h at 37°C with shaking. The sucrose solution buffer was poured off, and the gel was rinsed twice with distilled water (DW). The gel was transferred to a Pyrex dish containing 50 ml TTC solution (0.1% 2, 3, 5-triphenyl-tetrazolium chloride and 0.5 M NaOH) and heated to a boil in an extraction hood until the color developed. After detection, the gel was washed with DW, placed in 10% glacial acetic acid, and dried (Shimma et al., 1997).

Two-dimensional PAGE and protein identification

Two-dimensional electrophoresis was performed as described

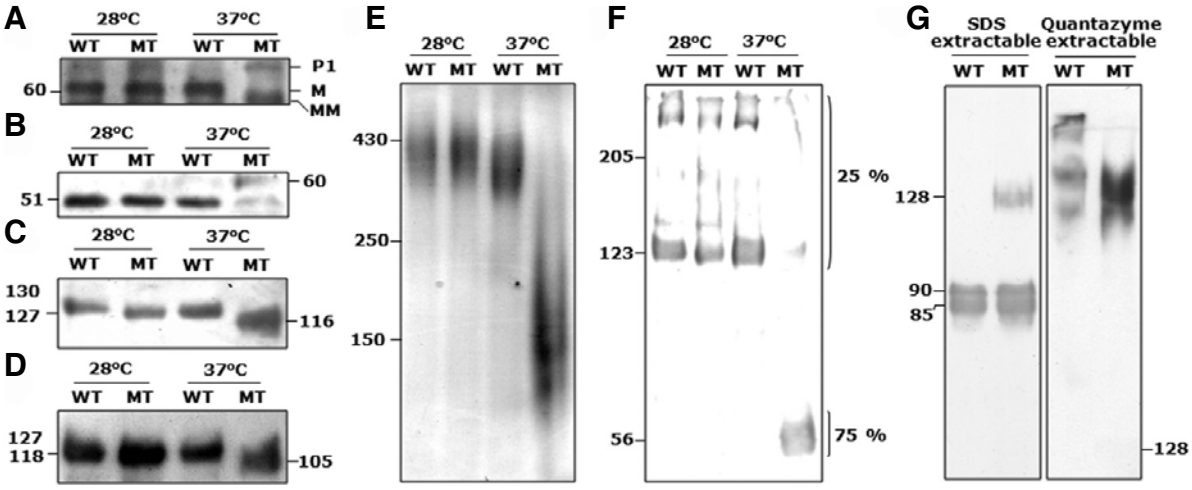

Fig. 1. Immunoblot analysis of marker proteins. Immunoblot analysis with anti-CPY antibody performed before (A) and after Endo H treatment (B). p1, ER core glycosylated form; M, normal mature form; MM, modified mutant mature form. (C) Immunoblot analysis of chitinase (Cts1p) with anti-Cts1p antibody. (D) Immunoblot analysis of Gas1p with anti-Gas1p antibody. Cts1p and Gas1p were extracted from cell walls using Laemmli buffer and analyzed by SDS-PAGE. (E) Activity staining of invertase. Invertase enzyme solution was prepared using YeastBuster and separated by 6% Gradi-Gel™ I. (F, G) Non-covalently linked Cwp1p and Sed1p proteins were extracted from the indicated strains using SDS extraction buffer. Protein samples were separated by SDS-PAGE and immunoblotted using anti-Cwp1p (F) or anti-HA antibodies (G). Covalently linked Sed1p was extracted by incubation with Quantazyme (β-1,3 glucanase) from the SDS-treated cell wall extracts. HA-tagged Sed1p was generated using the pRS424 vector (a gift from Dr. Shimoi). The migration of molecular size standards (kDa) is shown on the side of the image.

previously (Pardo et al., 1999). In brief, 200 μg protein mixed with 300 μl rehydration buffer (8 M urea, 2% CHAPS (3-[3-cholamidopropyl dimethylammonio]-1-propanesulfate), 10 mM DTT, 0.8% carrier ampholytes, and bromophenol blue) was applied onto immobilized pH gradient (IPG) strips (ReadyStrip: 17 cm, linear pH 3-10; Bio-Rad, USA) and passively rehydrated in a PROTEIN IEF Cell (Bio-Rad, USA) for 12 h. The isoelectrofocusing (IEF) was subsequently carried out as follows: 250 V for 15 min, 10,000 V for 4.5 h, and 90,000 Vhr. After the IEF, the IPG strip was equilibrated with 5 ml of the first equilibration buffer (6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, and 2% DTT) for 15 min, followed by 5 ml of the second equilibration buffer [6 M urea, 0.375 M Tris-HCl (pH 8.8), 2% SDS, 20% glycerol, and 2.5% iodoacetamide] for another 15 min. After the second-dimensional run was carried out on 12% SDS-PAGE, the gel was fixed with fixation buffer (40% ethanol and 10% glacial acetic acid) overnight and then sensitized with sensitizing buffer (15% ethanol, 0.125% glutardialdehyde, 0.2% sodium thiosulphate, and 3.4 g sodium acetate per 50 ml) for 30 min. The gel was carefully washed with DW 3 times for 5 min and the silver reaction was carried out on silver reaction buffer (2.5% silver nitrate and 0.0148% formaldehyde) for 20 min. After the silver reaction, the gel was washed twice for 1 min and developed in 0.025% sodium carbonate and 0.0074% formaldehyde until spots developed; the reaction was stopped by the addition of 0.365% EDTA. The silver-stained gel was scanned with the ProXPRESS Imaging System (PerkinElmer LAS, USA) and analyzed using INVESTIGATOR HT ANALYZER v3.01 (Genomic Solution, USA). Protein spots were excised and analyzed by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry. The protein sequence information was used to identify proteins in the National Center for Biotechnology Information (NCBI) database.

RESULTS AND DISCUSSION

Impaired glycosylation of marker proteins

Carboxypeptidase Y (CPY) - a protein marker used to monitor trafficking to the vacuole - exists as 3 different species: the ER-core glycosylated (p1, 67 kDa), Golgi-modified (p2, 69 kDa), and vacuolar mature forms (M, 61 kDa) (Johnson et al., 1987). To analyze the effect of the *ret1-1* mutation on the post-translational modification of CPY, we performed an immunoblot analysis with an anti-CPY antibody. Normal mature CPY (M) was detected in the wild type at both permissive and non-permissive temperatures. In contrast, a modified mature form with increased mobility (designated MM, 59 kDa) and the p1 form were detected in *ret1-1* cells grown at the non-permissive temperature (Fig. 1A). The signal intensity of the MM form was about 3 times greater than that of the p1 form. The presence of the p1 form of CPY indicates that the anterograde transport of CPY from the ER to the Golgi is partially impaired by the *ret1-1* mutation. This is consistent with a previous report describing this particular phenotype of the *ret1-1* allele (Schroder-Kohne et al., 1998). The MM form from the mutant cells migrated at the same rate as the wild type M protein following *N*-glycan cleavage by endoglycosidase H (Fig. 1B), indicating that the reduced size of the modified mature form of CPY in the mutant is not caused by proteolytic cleavage but by hypoglycosylation. The activity staining of invertase, which contains 14 potential glycosylation sites but no *O*-glycosylation sites (Ballou, 1990), revealed that at the non-permissive temperature, the *ret1-1* cells contained invertase proteins with much lower apparent molecular weights than those of the wild type (Fig. 1E). These results strongly indicate that the *ret1-1* mutation impairs the *N*-glycosylation of secretory proteins during transport from the ER to the vacuole and the cell surface.

The modification of chitinase (Cts1p) is a good indicator of the degree of the *O*-glycosylation of cell walls (Kuranda and

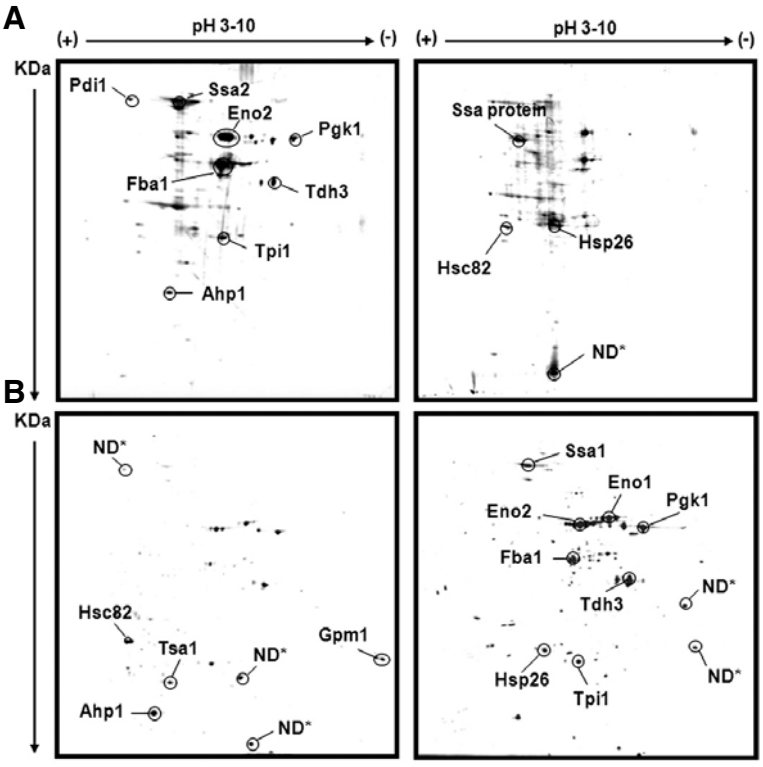

Fig. 2. Two-dimensional gel electrophoresis of proteins. The two-dimensional gel electrophoresis of the SDS-extractable cell wall proteins (A) and the proteins from culture filtrates (B) of wild type (left panels) and mutant cells (right panels) grown at non-permissive temperatures with 1.2 M sorbitol. Representative results from 3 independent experiments are presented. Protein spots were analyzed by MALDI-TOF identification of protein spots. Brief descriptions of the proteins identified are presented in Table 2.

Robbins, 1991). To assess the *O*-glycosylation of Cts1p, western blot analysis was performed with an anti-Cts1p antibody (Fig. 1C). At both permissive and restrictive temperatures, Cts1p extracted from *ret1-1* cells migrated faster than Cts1p derived from wild-type cells. Thus, the *O*-glycosylation of cell wall proteins is perturbed by the *ret1-1* mutation.

Altered post-translational modification of cell wall proteins

Gas1p is *N*-glycosylated, extensively *O*-glycosylated, and receives a glycosylphosphatidylinositol (GPI) anchor at its carboxyl terminus during its maturation along the secretory pathway. The transfer of carbohydrates and glycolipids to the immature 105 kDa protein starts in the ER and continues in the Golgi, resulting in a ~125 kDa mature form of Gas1p (Liu et al., 2006). As shown in Fig. 1D, immunoblot results for Gas1p from *ret1-1* cells are similar to those for Cts1p, suggesting that post-translational modification of Gas1p is also impaired. Sutterlin et al. (1997) found that Gas1p in *ret1-1* cannot be transported from the ER to the Golgi at a non-permissive temperature; this discrepancy may be explained by the different growth conditions used in the 2 studies. Sutterlin et al. (1997) grew *ret1-1* mutant cells under precisely restrictive conditions until the cells stopped growing and were about to undergo lysis. In contrast, the *soo1-1/ret1-1* mutant cells used in the present study were grown with 1.2 M sorbitol as an osmotic stabilizer that permitted the cells to continue growing even at non-permissive temperatures.

Cwp1p is a GPI-modified protein (molecular mass, ~55 kDa) covalently linked to the cell wall and is solubilized by β-1,3 glucanase. In addition to GPI-dependent incorporation, Cwp1p can be alternatively linked to the cell wall in a mild alkaline-sensitive manner (Kapteyn et al., 2001). Since the C-terminal region of Cwp1p has a region that is highly *O*-glycosylated (Pardo et al., 1999), we hypothesized that the *ret1-1* mutation might affect the glycosylation of Cwp1p as well as its linkage to the cell wall. To address this question, immunoblot assays were carried out on SDS-extractable cell wall proteins. As shown in Fig. 1F, the 123 kDa form of Cwp1p was detected in wild type cells at both temperatures and in the mutant at the permissive temperature. In contrast, when *ret1-1* cells were grown at a non-permissive temperature, Cwp1p was predominantly detected in a ~56 kDa hypoglycosylated form, which constituted 75% of all forms of Cwp1p detected in *ret1-1* under these conditions. This shows that at non-permissive temperatures, the *ret1-1* mutation perturbs Cwp1p glycosylation. In addition, most of the 123 kDa Cwp1p in *ret1-1* is not covalently attached to cell wall carbohydrates.

SED1 encodes a 34 kDa polypeptide that is rich in threonine and serine. Like other structural cell wall proteins, it contains a putative signal sequence for the addition of a GPI anchor (Shimoi et al., 1998). Wild type and mutant cells expressing HA-tagged Sed1p were cultured at a non-permissive temperature, and the SDS-extractable cell wall proteins were separated (Fig. 1G). Sed1p - a covalently linked cell wall protein - should only be solubilized by β-1,3-glucanase; however, the lighter forms of HA-tagged Sed1p migrated to the 85 and 90 kDa positions in both wild type and *ret1-1* mutant cells. An SDS-soluble 128 kDa band was detected only in the *ret1-1* mutant (Fig. 1G, left panel). After extraction with SDS, the covalently linked Sed1p was then extracted from the SDS-insoluble fraction by β-1,3-glucanase and separated on SDS-PAGE. As shown in Fig. 1G (right panel), the β-1,3-glucanase-extracted Sed1p from *ret1-1* migrated faster than that from the wild type. Thus, even the Sed1p that was covalently linked to cell wall carbohydrates in the *ret1-1* mutant was hypoglycosylated.

In *S. cerevisiae*, mannoproteins can be linked with other cell wall polymers in numerous ways; in some cases, the polymannose structure is thought to form a direct link with β-1,3-glucan chains, some proteins are linked through a remnant of their GPI anchor, and others appear to be directly linked to β-1,3-glucan through their protein moiety (Leasge and Bussey, 2006). There-

Table 2. Proteins identified from 2D-PAGE of the SDS-extractable cell wall proteins and the proteins from cultured medium

Protein name	Spot no.*	Family	NCBI entry	Z score	No. peptides matched	Seq. c. (%)**	Database		This study	
							pI	MW	pI	MW
Pdi1 (protein disulfide isomerase)	1	Protein folding	6319806	2.31	7	16	4.4	58.55	4.3	77
Ssa2 (heat shock protein)	2	Heat shock protein	6323004	2.36	14	23	4.9	69.62	5.2	73
Ssa protein (heat shock protein)	9	Heat shock protein	6323004 /6319314	2.33	8	11			5.0	56
Ssa1 (heat shock protein)	20	Heat shock protein	6319314	2.40	17	28	5.0	69.92	5.0	69
Eno2 (enolase)	3	Glycolysis	6321968	2.17	12	33	5.7	46.95	6.5	56
	21			2.25	13	35			6.0	50
Eno1 (enolase)	22	Glycolysis	6321693	2.38	15	40	6.2	46.86	6.7	52
Pgk1 (3-phosphoglycerate kinase)	4	Glycolysis	13786936	2.31	17	46	7.8	44.60	8.0	55
	23			0.50	3	11	7.8	44.60	7.8	50
Fba1 (fructose-bisphosphate aldolase II)	5	Glycolysis	6322790	2.32	7	24	5.5	39.89	6.4	45
	24			2.06	6	25			5.8	45
Tdh3 (glyceraldehyde-3-phosphate dehydrogenase3)	6	Glycolysis	6321631	1.50	6	17	6.5	35.84	7.5	39
	25			2.39	8	26			7.3	39
Tpi1 (triose phosphate isomerase)	7	Glycolysis	230405	2.28	10	37	5.7	26.76	6.5	25
	28			2.34	9	44			6.0	25
Ahp1 (alkyl hydroperoxide reductase)	8	Detoxifying	6323138	0.70	3	20	5.0	19.27	5.0	16.5
	18			2.35	6	34			4.5	19
Hsc82 (chaperonin)	10	Heat shock protein	6325016	0.97	5	6	4.8	81.39	4.8	26
	14			1.31	6	9			3.8	27
Hsp26 (heat shock protein)	11	Heat shock protein	6319546	1.68	5	32	5.3	23.86	5.9	27
	27			2.15	7	49			5.3	26
Tsa1 (chaperonin)	15	Detoxifying	6323613	2.09	5	32	5.0	21.69	5.0	22
Gpm1 (phosphoglycerate mutase)	17	Glycolysis	6322697	1.74	6	32	8.3	26.83	10.0	27

*Spot number 12, 16, 19, 26, and 29 were not identified.
**Sequence coverage

fore, defective glycosylation of cell wall mannoproteins may cause defects in cell wall integrity. In this study, the cell wall proteins, Cwp1p and Sed1p, were readily released with SDS, indicating that *ret1-1* exhibits defects in the post-translational modifications of cell wall proteins.

Enhanced secretion of cell wall-bound proteins into culture medium

To identify other cell wall proteins whose linkages (and thus location) are affected by the *ret1-1* mutation, the soluble fraction of SDS extracts were separated by 2D-PAGE (Fig. 2, upper panels). Analogous to the 2D map of proteins secreted by the protoplasts of *S. cerevisiae* cells during cell wall regeneration (Pardo et al., 1999; 2000), most cell wall proteins identified were glycolytic enzymes (Eno2, Pgk1, Fba1, Tdh3, Tpi1) or heat-shock proteins (Ssa2, Hsc82, Hsp26) (Table 2). Several glycolytic enzymes and heat-shock proteins are also abundant in the fractions of non-covalently bound proteins released by detergents and reducing agents (Insenser et al., 2010; Pitarch et al., 2002) and are thus already classified as cell wall components in *Candida albicans* (Angiolella et al., 2002; Eroles et al., 1997; Hernaez et al., 2010; Kelly and Kavanagh, 2010) and more recently in *S. cerevisiae* (Insenser et al., 2010; Kim and Park, 2004; Pardo et al., 1999; 2000). It is interesting that these proteins do not have the conventional N-terminal signal sequence found on most secretory proteins; therefore, it is proposed that they use an alternative mechanism of secretion instead of the classical ER-to-Golgi secretory pathway (Lopez-Villar et al., 2006; Nombela et al., 2006; Pardo et al., 1999). At present, 17 N-terminal secretion signal-lacking proteins are identified at the cell surface of *S. cerevisiae* and are classified into 3 groups on the basis of different cytoplasmic function: glycolysis, chaperone, and several other processes including redox homeostasis (Nombela et al., 2006). At wild type cell walls, proteins involved in glycolysis were detected exclusively, whereas heat shock proteins with the capacity to function as chaperones were abundant at the cell wall of the *ret1-1* strain. Although proteins were prepared from the cells grown at 37°C, it is noteworthy that an ER-chaperone, protein disulfide isomerase (Pdi1), and wild-type and cytoplasmic chaperones, Hsp26 and Hsc82, in the *ret1-1* mutant were newly identified as non-covalently bound cell wall proteins in this study. Most interestingly, the *ret1-1* strain secreted the majority of cell wall proteins found in the wild type into the growth medium at a high rate (Fig. 2, lower right panel); this indicates that the localization of secretion signal-lacking proteins delivered to the cell wall via the alternative secretory pathway is affected by the *ret1-1* mutation at the non-permissive temperature.

Aberrant retrograde transport of ER- resident proteins

From results described in the previous sections, it is evident that the temperature-dependent osmo-sensitivity of the *ret1-1*

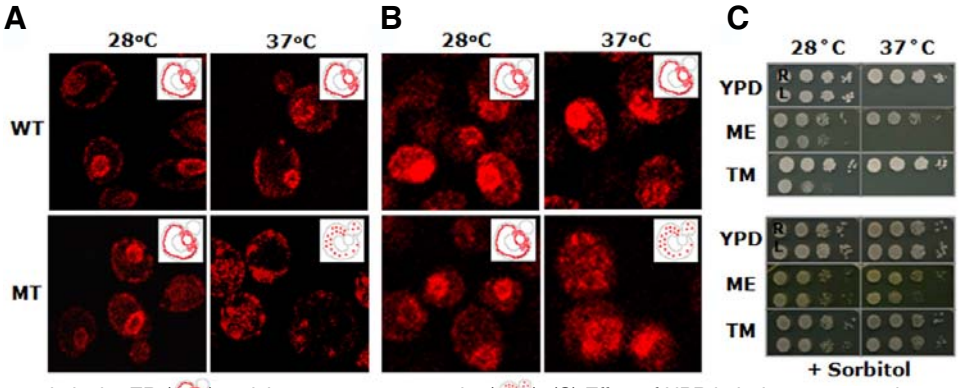

Fig. 3. Localization of the ER resident proteins (Wbp1p and Pmt2p) and sensitivity toward UPR-inducing agents. (A) Microscopic image of Wbp1p and (B) Pmt2p. The C-terminus of each protein was tagged with RFP on using the tag-marker cassette, pFA6a-mRFP-KanMX6. All strains were grown on YPD plates with 0.1 mg/ml G418; D-sorbitol was added to the plates used at 37°C. Cartoons in the inserts depict the localization of protein in the ER ( ) and the punctuate composite ( ). (C) Effect of UPR-inducing agents on the growth of strains. R, wild type; L, *ret1-1* mutant; YPD, YPD without UPR-inducing agent; ME, YPD with β-mercaptoethanol (10 mM); TM, YPD with tunicamycin (0.47 μM).

mutant cells is caused by post-translational defects including the hypoglycosylation of cell wall proteins. Therefore, we investigated the post-translational modification of glycosyltransferase subunits in the ER. Schröder-Kohne et al. (1998) used an invertase-Wbp1p chimera to confirm that α-COP is responsible for the retrograde transport of Wbp1p. Wbp1p contains a short cytoplasmic carboxyl-terminal domain with the ER retrieval motif, KKXX. Sequence analysis using PSORT II (http://psort.ims.u-tokyo.ac.jp) predicts that Pmt2p has an ER-retrieval KKXX-like motif in its exposed carboxyl-terminal domain (data not shown) and that its retrograde transport might also be carried out by COPI vesicles. Recently, the ER-localization of Wbp1p and Pmt2p was confirmed by a global analysis of protein localization using yeast strains expressing chromosomally tagged GFP fusion proteins (Huh et al., 2003). Thus, the defective protein *O*-glycosylation in the *ret1-1* mutant can be explained by the impaired retrieval transport of glycosyltransferase proteins to the ER.

To test the effect of the *ret1-1* mutation on the intracellular localizations of Wbp1p and Pmt2p, RFP tags were integrated downstream of the endogenous genes coding these proteins and the fluorescent products were visualized by confocal microscopy. As shown in Fig. 3, both wild type cells and *ret1-1* grown at a permissive temperature exhibited Wbp1p and Pmt2p localization at the ER, which is identical to the image of these 2 proteins in the yeast GFP fusion localization database (http://yeastgfp.yeastgenome.org/index.php). In contrast, in *ret1-1* cells grown at a non-permissive temperature, localization impairment of either Wbp1-RFP or Pmt2-RFP was detected; these proteins appeared to not entirely accumulate in the ER but rather in the punctuate composites, which may include the Golgi and other organelles. One possible explanation for these results is that the mutant Ret1-1 coatomer does not recognize the KKXX signals of Wbp1p and Pmt2p (Letourneur, et al., 1994; Schröder-Kohne et al., 1998), resulting in a failure of these cargo proteins to be embedded into COPI vesicles. In addition, if the *ret1-1* α-, β′-, and ε-COP subcomplexes of the COPI vesicle are defective, this leads to aberrant COPI vesicle formation in this particular mutant (unpublished results). These defects in *ret1-1* may adversely affect the retrieval of ER resident proteins, such as Pmt2p and Pmt4p, and in turn, the glycosylation of *in vivo* substrates such as Cts1p and Gas1p.

Sensitivity to UPR-inducing drugs

N-linked glycans added to nascent polypeptides in the ER are important for correct protein folding. The accumulation of unfolded proteins causes the activation of an adaptive signaling cascade known as the unfolded protein response (UPR), which acts to increase the folding capacity of the ER and to decrease the folding load by blocking ER-associated degradation (ERAD) and protein synthesis (van Anken and Braakman, 2005). If the protein-folding defect is not resolved, the UPR shifts to more extreme measures including cell-cycle arrest and eventual cell death (Malhotra and Kaufman, 2007). Since the *ret1-1* mutation hampers the retrieval of ER resident proteins and consequently *N*- and/or *O*-glycosylation in the ER, we expect the response of this mutant to UPR-inducing drugs to be hampered as well. As shown in Fig. 3C (upper panel), even at permissive temperatures, the *ret1-1* mutant exhibited increased sensitivity to the UPR-inducing drugs, tunicamycin (TM; an *N*-glycosylation inhibitor) and β-mercaptoethanol (β-ME; a reducing agent that blocks disulfide formation). However, the sensitivity of *ret1-1* to these drugs was completely reversed by the addition of sorbitol to the medium except in the case of β-ME treatment at a non-permissive temperature (Fig. 3C, lower panels). These results are consistent with a recent report stating that defects in *N*-glycosylation caused by *ost* mutations along with the treatment of wild-type strains with TM at high temperatures trigger yeast apoptosis; however, the induced apoptosis of *ost* mutants cannot be prevented by osmotic support (Hauptmann et al., 2006), while the TM-induced cell death of the *ret1-1* mutant was protected by the presence of sorbitol. These results indicate that the adverse effects of the UPR-inducing agents on the *ret1-1* mutant are primarily exerted on the proteins required for cell wall integrity and that TM alone is sufficient to induce the cell death of the *ret1-1* mutant without a temperature stimulus. In *S. cerevisiae*, Pmt2p-dependent *O*-mannosylation affords protection from ERAD by solubilizing the aberrant protein overflow from the ERAD pathway and reducing the load on ER chaperones. Pmts are also involved in the cell wall integrity pathway via the *O*-mannosylation of PKC1 pathway sensor proteins, such as Wsc1p, Wsc2p, and Mid2p, in response to external stress (Goto, 2007). The UPR and ERAD pathways are required for proper cell wall construction; ER and cell wall stress responses are coordinately regulated by cell wall integrity and UPR signaling pathways to protect cells against these related cellular stresses (Scrimale et al., 2009). Thus, reduced *O*-mannosylation by Pmt2p malfunction in the *ret1-1* mutant at non-permissive temperatures may result in an unspecific cleavage of the PKC1 pathway sensor proteins, which in turn results in impaired activation of the cell integrity pathway in response to cell wall stress.

The different effects of TM and β-ME on the *ret1-1* mutant at non-permissive temperatures can be explained by the fact that

the defect in *N*-glycosylation was induced by the *ret1-1* mutation at a non-permissive temperature like TM treatment. However, changes in the redox status of the ER lumen caused by β-ME treatment could act as an additive insult that may cooperatively cause irreversible cell death of the *ret1-1* mutant at non-permissive temperatures.

In summary, our data suggest that impaired glycosylation caused by the mislocalization of ER resident proteins is responsible for the alterations in the secretome and the increased sensitivity of *ret1-1* mutant cells to ER stress. Despite the extensive functional information that is available for many yeast genes, very little is known about the *RET1* for α-COP and its role in protein secretion. The present study provides new insights into the function of Ret1p/α-COP in protein secretory pathways, cell wall integrity, and ER stress responses.

ACKNOWLEDGMENTS

We thank H. Shimoi (NRIB, Japan) for providing the Cwp1p antibody and the pRS424 vector, as well as W. K. Huh (SNU, Korea) for providing the pFA6a-mRFP-KanMX6. This work was supported by a Korea Research Foundation Grant (KRF-C00170).

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